

Universidade de Lisboa
Faculdade de Ciências
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Different Behaviours elicited by CO₂ in fruit fly larvae

Nuno Pimpão Santos Martins

Mestrado em Biologia Evolutiva e do Desenvolvimento

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List of abbreviations

Or	Olfactory receptor
ORN	Olfactory receptor neuron
AL	Antennal Lobe
MB	Mushroom bodies
LH	Lateral Horn
PN	Projection neuron
LN	Local Interneuron
Gr	Gustatory receptor
GRN	Gustatory receptor neuron
SOG	Subesophageal Ganglion
DO	Dorsal Organ
TO	Terminal Organ
VO	Ventral Organ
DPS	Dorsal Pharyngeal Sensilla
VPS	Ventral Pharyngeal Sensilla
PPS	Posterior Pharyngeal Sensilla
PI	Preference Index
C-S	Canton-Special wild type flies
dH ₂ O	distilled Water

Abstract

CO₂ molecules are present almost everywhere and have many biological roles, special as environmental cues or metabolic products of animals. Due to its importance, many insect species are able to sense its presence and concentration. In animals this molecule elicits appetitive behaviours, like moths and mosquitoes. On others, like fruit flies of the *Drosophila melanogaster* species, this molecule strong repulsive behaviours.

There are specific CO₂ receptors with the role of detecting this gas. However, their localization is different, according to the behaviours it elicits. Receptors in moth and mosquitoes, are present in the maxillary palps, while in fruit flies, receptors are in the antennae.

However, it has been reported that CO₂ can trigger attractive behaviours in *D. melanogaster*, but CO₂ is being released by a liquid solution. This appetitive behaviour is associated with the presence of several microorganisms that release CO₂ as a product of their metabolism. Therefore, these solutions are sensed by flies as appetitive. Although, CO₂ is not being sensed by the antennae, like its gas phase. Since, CO₂ is released by a liquid, it is being sensed by specific gustatory neurons in the proboscis.

Therefore, CO₂ can elicit different behaviours in the same insect. A possible reason for this different are the different projections of the sensory system, olfaction and taste. But those projections are hard to study in adults of *D. melanogaster*, therefore, larvae were used, because they are much simpler. However, larvae show aversive behaviours to CO₂ in its gas phase, but the behaviour to carbonated solutions is not known. In this work was seen that larvae can feed on liquids and effectively choose between them, and in the presence of a carbonated solution, they show appetitive behaviours like adults. Therefore, we can characterize the projections of the gustatory and olfactory system to associate different brain regions with different behaviours.

Keywords: CO₂, feeding, appetitive and aversive behaviours, *D. melanogaster* larvae, neuronal and sensory structures

Resumo

A molécula de CO₂ tem uma grande importância biológica, porque é bastante usada pelos mais variados insectos como um sinal olfactivo, mas também é um dos principais produtos do metabolismo. Isto, permite que vários animais o usem como sinal olfactivo para as mais variadas funções.

Vários insectos como os mosquitos e as traças usam o CO₂ como sinal olfactivo para procurar fontes de alimento. As traças usam este composto para descobrirem plantas onde se alimentam, visto que ele é libertado como produto do metabolismo, dando indicações da sua posição à traça. Os mosquitos por seu lado, usam o CO₂ para detectarem animais, porque se alimentam de sangue. Os animais onde os mosquitos se alimentam libertam plumas de CO₂ pela respiração, o que lhes permite localizar o hospedeiro. Portanto, quando na presença de CO₂, tanto a traça como o mosquito apresentam comportamentos atractivos, visto serem atraídos pela fonte de CO₂. Por outro lado, a mosca da fruta, *Drosophila melanogaster*, apresenta comportamentos diferentes quando detecta CO₂. Os comportamentos apresentados por moscas da fruta são aversivos, o que faz com que fujam da fonte de CO₂. O que poderá estar implicado neste comportamento é a presença deste gás no odor de stress que as moscas libertam quando em condições adversas. No entanto, em todos os casos há receptores específicos para o CO₂, embora estejam localizados em zonas diferentes. No caso das traças e dos mosquitos, os receptores estão em estruturas olfactivas associadas à alimentação, os Palpos Maxilares. Na mosca da fruta, os receptores encontram-se nas antenas, que são órgãos sensoriais associados ao olfacto.

Contudo, foi descoberto por Fischler *e tal.* (2007), que as moscas apresentam outros comportamentos na presença de CO₂. Agora, em vez de comportamentos aversivos, a mosca apresenta comportamentos atractivos. Mas neste caso não é apenas para o CO₂ e sim para uma solução q liberta este gás. Foi descoberto, no entanto, que o sistema sensorial que detecta o CO₂ nestas condições, é o sistema gustativo, por células específicas que apenas detectam CO₂ libertado por carbonação. Isto está associado a soluções onde há

microrganismos, o que tornam a solução mais atractiva e com valor nutricional. Carbonação é uma reacção onde HCO_3^- é convertido em CO_2 e H_2O . Assim, esta solução liberta CO_2 , imitando soluções onde há microrganismos. Portanto, as moscas deverão associar a libertação de CO_2 à presença de uma solução nutritiva. É importante ter em consideração que a mesma molécula de CO_2 podem desencadear dois comportamentos diferentes no mesmo insecto. O que indica que as projecções nervosas deverão ser a causa para esta diferença no comportamento.

Assim, caracterizando as projecções nervosas de ambos os sistemas sensoriais, seria possível associar regiões cerebrais a comportamentos como atracção e aversão.

Contudo, as estruturas sensoriais não estão completamente descritas, tanto a nível gustativo como a nível olfactivo. Devido ao elevado número de células nervosas existentes no cérebro da mosca da fruta. No entanto, um possível alternativa à mosca da fruta, é a sua larva, que possui as mesmas estruturas olfactivas e gustativas mas em número reduzido. O que facilitaria a caracterização das tais estruturas sensoriais. É sabido que as larvas da mosca da fruta apresentam comportamentos aversivos para o CO_2 no estado gasoso, tal como os adultos. Mas não se sabe que comportamentos apresentam na presença de soluções carbonatadas e q libertam CO_2 .

Portanto, será necessário caracterizar estes comportamentos. Mas para isso é necessário criar um setup experimental onde isso seja possível. Visto que as experiências de alimentação feitas em larvas têm sido feitas com comida sólida. Assim, é necessário ver se as larvas conseguem alimentar-se de soluções líquidas. O que se comprova com este estudo. Posteriormente, é visto neste trabalho que as larvas, além de conseguirem alimentar-se em líquidos, também conseguem escolher eficazmente entre duas soluções diferentes. O que leva à experiência de maior importância. Depois de sabermos que as larvas se podem alimentar em líquidos e até conseguem escolher eficazmente entre eles, testamos os comportamentos para soluções carbonatadas.

O resultado destas experiências indica que as larvas são atraídas por soluções carbonatadas, tal como os adultos. O que sugere que os mesmos receptores gustativos estão presentes em larvas e que estas associam a solução

carbonatada à existência de microrganismos. No entanto, outra linha de moscas foi utilizada para despistar qualquer contribuição olfactiva do CO₂ para a experiência. Esta nova linha é incapaz de detectar este gás por meios olfactivos. Com estas experiências vimos que não há contribuição olfactiva do CO₂ para o comportamento, o que indica que o gás libertado pelas soluções carbonatadas não é suficiente para alterar o comportamento das larvas.

Portanto, é possível utilizar as larvas para estudar e caracterizar as projecções nervosas para CO₂, que desencadeia comportamentos diferentes quando detectado por sistemas sensoriais diferentes.

Sendo assim possível estudar que regiões cerebrais são responsáveis pelo desencadear destes comportamentos.

Palavras Chave: CO₂, alimentação, comportamentos apetitivos e aversivos, *D. melanogaster*, estruturas nervosas e sensoriais

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Introduction

Every living organism is interacting with its environment and the surrounding living beings, with very important associations and interactions. In order to get information with the surrounding environment, the organism has to use its senses, which translate environmental cues into information able to be interpreted by the brain.

Sensory systems are very important in understanding the surrounding environment, like the olfactory and gustatory systems that sense cues in the most variable environments. The olfactory system detects a broad range of cues with different functions. Some roles consist of detecting food sources, toxic or dangerous compounds or even sexual partners, etc. The gustatory system has similar roles, it can detect a wide range of palatable or nauseating foods cues determining what to eat, and is important in sexual receptivity as well (Moon *et al.*, 2009; Koganezawa *et al.*, 2010). However, there is a difference between these two systems, the olfactory system is able to detect long ranged cues, while the gustatory system detects close ranged cues.

Even though sensory systems integrate specific cues with more than one function, in the wild, cues cannot be separated and all information is integrated at the same time. Therefore, behaviours can be triggered by a single cue or by a combination of cues acting at the same time.

CO₂ is one of the most important olfactory cues because it is present everywhere and plays lots of roles and functions. It can be integrating part of metabolic processes, pH equilibrium reactions and energy acquisition processes. Since its a major product of metabolic reactions, it can be used by living organisms for detection of many metabolic events.

CO₂ as an olfactory cue

Many animals can detect CO₂ and use as an olfactory cue. Mosquitoes and moths can detect CO₂ by specific receptors in the olfactory pieces near the proboscis, the maxillary palps, and when detected it elicits appetitive behaviours towards the source. Moths use CO₂ to detect food sources, like plants they feed

on, because they release it as a product of their metabolism (Guerenstein *et al.*, 2004; Cayirlioglu *et al.*, 2008). While mosquitoes' food source are animals, because they release plumes of CO₂ in their breathing and this acts as an appetitive olfactory cue. Since mosquitoes feed on blood, they detect their food sources by sensing CO₂ (Distler and Boeckh, 1997; Takken and Knols, 1999; Cayirlioglu *et al.*, 2008).

On the other hand, insects like *D. melanogaster* use CO₂ as different olfactory cues. Instead of showing appetitive behaviours, like mosquitoes or moths, they show high aversive behaviours when detecting this gas (Suh *et al.*, 2004; Faucher *et al.*, 2006). A possible reason for this behaviour is the high anaesthetic power of CO₂, although, this only happens with very high levels of CO₂. In addition, when flies are stressed, they release a stress odorant that has CO₂, although there are other unknown compounds that elicit aversive behaviours (Suh *et al.*, 2004).

In all cases above, specific receptors act in the detection of CO₂, however, the organs where they are present is different. The receptors for CO₂ in moths and mosquitoes are in the maxillary palps (Distler and Boeckh, 1997; Guerenstein *et al.*, 2004; Cayirlioglu *et al.*, 2008). These olfactory organs are mainly associated with feeding behaviours and it is normal to see appetitive behaviours towards compounds detected by receptors on these organs. In fruit flies, the olfactory receptors for CO₂ are in the third antennal segment, where most olfactory receptors are. Different localization of CO₂ receptors reflect the change in using CO₂ as an olfactory cue (Cayirlioglu *et al.*, 2008).

Organization of olfactory structures in *D. melanogaster*

Olfactory structures of *D. melanogaster* are well known and described. Odours bind to specific olfactory receptors (Ors), or receptor-binding proteins, housed in sensory neurons present either in the antennae or the maxillary palps, called the Olfactory Receptor Neurons (ORNs). ORNs are housed in hair-like structures called sensilla with clusters of two to four neurons, depending on the type of sensilla (Couto *et al.*, 2005). These neurons project to a structure called the Antennal Lobe (AL), where the first information processing is done. All ORN

are specific for an odour type and only have one Or. In addition to the specific Or, ORNs have a co-receptor called Or83b, necessary to function properly.

CO₂ specific receptor neurons have been identified in *D. melanogaster*, with two olfactory receptors, the Gr21a and Gr63a (Suh *et al.*, 2004; Jones *et al.*, 2007; Kwon *et al.*, 2007). These neurons are housed in a sensilla with three other olfactory neurons. Interestingly, CO₂ sensing neurons lack Or83b, suggesting a different role as olfactory receptor neurons. Another interesting fact is the inability to function properly without the presence of both CO₂ receptors (Suh *et al.*, 2004; Jones *et al.*, 2007; Kwon *et al.*, 2007).

The AL structure is composed of 43 glomeruli with specific affinity for odours, because ORNs project to a single glomeruli (Vosshall *et al.*, 2000; Fishilevich *et al.*, 2005). Therefore, ORN of the same type project to a single glomeruli of that same type. However, the glomerulus where Gr21a/Gr63a neurons project to, is slightly different from the other glomeruli. Because it is outside of the AL, below every other glomeruli, and can be easily distinguishable. Afterwards, the information from the AL is relayed to higher order structures called the Mushroom Bodies (MB) and the Lateral Horn (LH), where information processing goes on and some memory and associations are formed (Clyne *et al.*, 1997; Laissue *et al.*, 1999; Gao *et al.*, 2000; Vosshall *et al.*, 2000; Marin *et al.*, 2002; Couto *et al.*, 2005; Fishilevich *et al.*, 2005; Faucher *et al.*, 2006).

Communication between the AL and the MB and LH is done by a subset of neurons called Projection Neurons that connect glomeruli in the AL to the MB and LH. This is the extent of known connections in the olfactory brain because from here on the complexity of the brain gets bigger and the connections are difficult to characterize and isolate. Although, some attempts to characterize higher order regions have been made (Cachero *et al.*, 2010).

CO₂ elicits aversive behaviours in *D. melanogaster* when detected in high concentration (Suh *et al.*, 2004; Faucher *et al.*, 2006), however, when it is combined with 2,3-butanedione, a compound released by ripening bananas, the aversive behaviours are inhibited (Turner and Ray, 2009). This means that interaction of a combination of cues cannot be excluded and affects the final behaviour. The effect of a combination of cues is not the sum of their effects.

Inhibition and excitation by Local Interneurons (LNs) was discovered in the AL and is a possible explanation for the inhibition seen in Gr21a neurons (Huang *et al.*, 2010). This interaction between LNs and other neurons in the AL may give a bigger olfactory discrimination when detecting more than one olfactory cue at the same time, preventing a situation of misinterpretation of olfactory cues.

CO₂ as a gustatory cue

Until now, CO₂ has always been seen as an aversive molecule to *D. Melanogaster*, because the only known behaviour to CO₂ was elicited by high concentrations of CO₂ and resulted in aversive responses.

However, Fischler *et al.* (2007) saw that CO₂ has another role as a molecular cue. They saw through experimental assays that adult flies from *D. melanogaster* show appetitive behaviours to a solution that releases CO₂. Fruit flies prefer to feed on a solution with CO₂ release by carbonation as opposed to a plain solution with no release. Interestingly, the system where CO₂ molecules are detected is different. Fruit flies show appetitive behaviours to these carbonated solutions that are sensed by the gustatory system. Carbonation reactions are present in many metabolic events, and one of them is a pH equilibrium reaction. In carbonated solutions with low pH levels, there is conversion of HCO₃⁻ into H₂O and CO₂, increasing the pH level and releasing CO₂ to the surrounding environment. This reaction is present in living organisms, and blood is one of the examples.

Since CO₂ in its gas phase elicits aversive behaviours in *D. melanogaster*, it is surprising to see that a solution with CO₂ release triggers appetitive behaviours. These appetitive behaviours result as an association because many microorganisms release CO₂ as a metabolic product, and a solution releasing CO₂ suggests that microorganisms are present. Yeast, for example, is known to release CO₂ by its metabolism and, at the same time, is a known food source of fruit flies. Therefore, when flies get in contact with a solution that releases CO₂, they associate it with the presence of yeast or other organisms they feed on, indicating that this solution may have a higher nutritional value than a solution with no CO₂ release.

The receptors responsible for CO₂ detection and appetitive behaviours are found in the proboscis, in specific cells that only respond to CO₂ released from carbonation. The cell line that responds to CO₂ and carbonation in the gustatory system is called E409, because of the experimental assay where they were found. Fischler *et al.* (2007) saw that this cell line had concentration dependent responses toward solutions of carbonated water, revealing difference in receptors for CO₂. However, there is no evidence showing that CO₂ receptors in the olfactory system are concentration dependent, suggesting that E409 cells have gustatory receptors for CO₂ different from the olfactory system receptors.

Organization of the gustatory structures in *D. melanogaster*

Gustatory receptors (Gr) present in the gustatory system are, as the name suggests, receptors tuned to detect tastants. These molecules sensed by the gustatory system are associated to taste modalities. A taste modality is characterized by the kind of tastants that they gustatory receptor neurons (GRN) are tuned to detect. Tastes are separated into 5 categories like sugar, salt, bitter, sour and "umami". "Umami" is the taste responsible for amino acid detection. Each taste modality is represented differently in the taste sensory structure, by clusters of neurons tuned to detect one of the tastes. Carbonation receptors were seen to be different from the formerly described gustatory neurons, showing that this might be a new taste. Because they are a completely different type of cells that only respond to carbonation and are spatially separated from other GRNs.

Although the proboscis is the main gustatory structure in an adult fly, there are several places where other gustatory receptors are present, like the mouthparts, legs, wings and the ovipositor, although this last one is only present in female flies. Gustatory receptors have other roles besides feeding, they have important roles in receptivity as well, because they have an active role in courtship behaviour of flies, preventing them from courting flies of the same sex, or to identify female flies of the same species (Moon *et al.*, 2009; Koganezawa *et al.*, 2010).

To go into further detail of the structures of the gustatory system, it is needed to see what is known about sugar and bitter receptors neurons, since

these have been well described and heavily studied, in opposition to E409 cells. Although, projections of CO₂ GRNs are the same as other gustatory neurons. Like the olfactory system, there is a structure where the information from the gustatory receptor neurons is processed. That structure is called Subesophageal Ganglion (SOG) and is placed at the base of the brain. As in the olfactory system, this organ is directly connected to the sensory neurons, but it lacks a glomerular organization or subdivision into different regions. For example, sugar GRNs and bitter GRNs project to the same region in the SOG, although spatially segregated. However, the projections from different places, like leg neurons, project to different places in the SOG when compared to the same receptor neurons from the proboscis. In other words, sugar sensing neurons from the leg project differently from sugar sensing neurons from the proboscis, although the receptors are the same (Thorne *et al.*, 2004; Wang *et al.*, 2004). This kind of differential projection from neurons with the same receptor does not happen in the olfactory system.

Another difference between the gustatory and the olfactory system is the number of receptors that GRN can have. GRNs can have more than one receptor and the olfactory system only has one receptor per neuron, besides Or83b. An example of these multiple receptor neurons is sugar sensing gustatory receptors that express several Gr for sugars. Even though GRNs have multiple receptors, they only show receptors for one taste modality, with no redundancy between neurons (Thorne *et al.*, 2004; Wang *et al.*, 2004).

However, as in the olfactory system, projections to higher order organs or structures are unknown due to high cellular number and complexity. Because of this it is hard to see where the sensory structures project to, increasing the difficulty to associate brain regions with different basic behaviours like attraction of repulsion.

Larvae are simpler than the adult but have similar organization

Since the major difficulty is the high number of cells and neuronal complexity of the brain, there is an alternative to adult *D. melanogaster*. Larvae from *D. melanogaster* can be useful to study neuronal projections and the fly

brain because they have smaller brains with a smaller number of neuronal cells. However, the structure of the brain is the same as the adult, with some exceptions.

At the olfactory level, larvae have fewer olfactory receptors (Ors) than the adult, therefore they do not have the same number of ORNs. Adult flies have up to 60 times more ORNs than larvae, revealing olfactory redundancy in the olfactory system (Ramaekers *et al.*, 2005). In larvae there is only one ORN per Or, in a total of 21 neurons. All the ORNs in larvae are in a single organ called the Dorsal Organ (DO), the main olfactory organ of larvae. However, this organ has both gustatory and olfactory function. The DO has the same role as the Antennae and Maxillary Palps, however it is much smaller and less redundant. At brain level, AL in larva has a similar organization as in the adult, with the difference that there are no glomeruli. However there is some clustering of cells into glomeruli-like structures (Fishilevich *et al.*, 2005; Ramaekers *et al.*, 2005). PNs are present in larvae as well and have the same structure and connections as the adult (Python and Stocker, 2002). In addition, CO₂ olfactory sensing neurons are present in larvae and project to the larval AL. This renders CO₂ sensation possible in larvae, as they show aversive behaviours when in presence of high concentrations (Faucher *et al.*, 2006). Therefore, all olfactory projections and structures at brain level are similar to the adult, although with a smaller number of cells.

At the gustatory level, larvae have a similar number of gustatory receptors (Grs) and a similar number of sensory neurons as well. The main difference between adults and larvae is the gustatory organs themselves. Larvae do not have proboscis, legs, wings or the same mouthparts, so their gustatory receptors are present in 6 different organs near or inside the digestive tract. Three of them are in the anterior most region of larvae and they are called the Dorsal organ (DO), Terminal organ (TO) and Ventral organ (VO). The other three are in the beginning of the digestive tract, more precisely in the pharynx, being called Dorsal Pharyngeal Sensilla (DPS), Ventral Pharyngeal Sensilla (VPS) and Posterior Pharyngeal Sensilla (PPS). However, at brain level the connections of the GRNs are the same as in the adult, sensory neurons connect to the SOG, but from here on the projections are unknown.

In terms of behaviour, larvae show aversive behaviours towards high concentration of CO₂, like the adults. However, no behaviours elicited by carbonated water solutions were described in larvae.

Since larvae are simpler and have the same olfactory structures as the adult, it is possible, or at least easier, to characterize projections to high order brain regions. But until now it is only known the effect of CO₂ in its gas phase in larvae, and not the effect of a carbonated solution and which behaviours larvae show in the presence of this solution. Therefore, studying behaviour to carbonated solutions in larvae can be very useful to study higher order brain regions and characterize them. This is easier in larvae for several reasons, they are smaller, and therefore their cellular number is smaller, as described above. Some authors even consider larvae as the elementary model for olfaction in *D. melanogaster* (Ramaekers *et al.*, 2005).

The same genetical tools can be used in larvae, like the Gal4-UAS system and other genetic tools, giving many possibilities in behavioural assays. Since they are almost transparent, it makes imaging in the brain and other tissues easier.

After studying the response towards a solution of carbonated water, it would be possible to study possible differences in higher order brain regions, only if the behaviours shown by larvae are the same as in the adult, showing aversive behaviours when CO₂ is detected by the olfactory system (Suh *et al.*, 2004; Jones *et al.*, 2007), and appetitive behaviours when it is sensed by the gustatory system (Fischler *et al.*, 2007). This would be a big step in characterization of sensory structures. That would be easier using a single molecule like CO₂ to elicit different behaviours.

Objectives

Larvae have been widely used for feeding behaviour assays. Although they have only been used in feeding assays with solid food. In order to study their behaviour to a solution of carbonated water, a setup with liquid solutions is needed. Therefore, it is needed to see if larvae can feed on liquid solutions. After seeing if they can feed on liquids, they might not effectively choose between different solutions. Then, this possibility has to be tested as well, to see if larvae can effectively choose between different solutions.

Afterwards, larvae will be tested for a preference towards a liquid carbonated solution, to see if they which behaviours are shown in the presence of these solutions. Although, a possible olfactory contribution of CO₂ may change the outcome of the behaviour assay. Therefore, this possibility will be tested for a possible change in the behaviour shown by larvae.

Methods

Populations

Two strains of flies from the *Drosophila melanogaster* species were used, a Canton-Special wild type strain and a Gr63a¹ mutant one. Both populations were reared and maintained in an incubator with constant temperature of 25°C, relative humidity of 70% and light-dark cycles of 12/12h. Fly stocks were kept in bottles with cornmeal based fly flood, with an average of 200 adult individuals per bottle.

For the experimental behaviour assays, third instar larvae with approximately 96 hours were used. The larvae were reared in the same conditions as the populations, in bottles with no adults so that the age of larvae was controlled and known.

Larvae rearing and collection

Flies were placed in new food bottles for 5 hours, in order to lay eggs and control larval age, so that they did not exceed 5 hours of difference between ages. Bottles with freshly laid eggs were then maintained at a 25°C incubator, as described above. Larvae were left to grow for 78h at 25°C, until they were old enough to begin the behavioural assay.

To collect larvae, a 17% Sucrose (Sigma) solution was prepared and used to separate larvae from the food, followed by PBS 1X to wash the remaining food and sucrose, repeated for another three times.

After washing, larvae were starved overnight, a starvation period around 15 hours, before the experimental assay. Starvation is a way to ensure that larvae feed during the experimental procedure. Larvae were kept and starved overnight in a 100x35mm Tissue culture dish (Sarstedt) filled with 30ml of Ultrapure™ Agarose (Invitrogen) 2,5% to retain humidity during the night period. Each of these Petri dishes had seven caps of 0,5ml eppendorf tubes (Sarstedt) to pose as containers, where dH₂O was placed to prevent dehydration.

Larvae were manipulated with Live Insect Forceps (Fine Science Tools) to prevent potential harm or damage.

Behavioural feeding assay

After overnight starvation, early third instar larvae (96 hours) were placed in 60x35mm Tissue culture dishes (Sarstedt) filled with 15ml of Ultrapure™ Agarose 2,5%. The Agarose gel was used to maintain a humid environment and to allow larvae free movement around the Petri dish. Each dish had two caps of 1,5ml eppendorf tubes (Sarstedt) as containers for the solution. These caps prevented the solution from mixing with the Agarose gel, since the later is permeable, even with such high concentration percentages. The gel percentage was high, so that larvae could not eat the Agarose gel, forcing them to feed on the liquid solution.

The following solutions were prepared on the same day as the experimental assay. With the exception of dyed solutions where Sucrose, Yeast Extract and Yeast were mixed. These dyed solutions were kept at 4°C in the dark to prevent degradation.

In early assays, Sucrose 0,1M, Yeast Extract and Yeast solutions were prepared, using Sucrose (Sigma), Yeast Extract (Conda labs) and Lyophilized Yeast from *Saccharomyces cerevisiae*, type II (Sigma), respectively. The later solutions, Yeast and Yeast Extract, were prepared for a final concentration of 0,05g/ml, as it seemed fit for the experimental assay. Dyed solutions were prepared with a red dye, Amaranth (Sigma) 0,05g/50ml concentration, or a blue dye, Indigo Carmine (Sigma) for a final concentration of 0,025g/50ml.

In later experimental assays, solutions of carbonated water were prepared in two different concentrations, 100mM and 200mM. These solutions were prepared mixing a solution of Sodium Bicarbonate with a buffer solution of Sodium Phosphate and adding one of the dyes afterwards. These solutions were newly made each day for the experimental assay. A carbonated solution was made with Sodium Bicarbonate (Sigma) dissolved in distilled water for a concentration of 200 or 400mM, depending on the final intended concentration. Buffer solutions were prepared with monobasic Sodium Phosphate, SigmaUltra

99% (Sigma) and dibasic Sodium Phosphate, SigmaUltra 99% (Sigma) dissolved in distilled water. Mixing different volumes of monobasic or dibasic Sodium Phosphate solutions resulted in different pH level. The final concentration of the Phosphate buffer added to the sodium bicarbonate solution was 1M, so that after mixing, the buffer had a 0,5M concentration. A 6,0 or a 6,5 pH buffer was prepared for the 100mM or 200mM bicarbonate solutions, respectively. The addition of a buffer solution was important to maintain low pH levels, as seen in Fischler *et al.* (2007). Because a certain pH level leads to a certain percentage of CO₂ species being converted from a Carbonate ion, corresponding to 70% and 40% CO₂ species for the 6.0 and 6.5 pH levels. In the end, there would be a 100 or 200mM carbonated solution with a 0,5M phosphate buffer with 6,0 or 6,5 pH level, respectively.

The experimental assays were done in a behaviour room with constant temperature and humidity. 30 larvae were placed for 2 hours in the dark. At the end of the assay, larvae were collected from the Petri dishes and scored for a coloured solution, or absence of such, in their digestive tract to calculate a Preference Index. Larvae were scored with the help of a stereoscope Leica M125.

After scoring larvae, a Preference Index was calculated by the following equation: $PI = (\text{Test solution} - \text{Control solution}) / \text{Total number of coloured larvae}$. Larvae with both colours were used for the Total of coloured larvae. Larvae with no colour in their digestive tract were discarded because they showed no preference to any solution, however, they were used as an indicator for the conditions of the experimental assays. Assays with fewer than 10 coloured larvae were excluded, because these assays had big variations and did not represent the real preference to a liquid solution. The Preference Index can vary between -1 to 1, where -1 represents aversive behaviours and a value of 1 represents appetitive behaviour.

Statistics

The statistical analysis of the data collected in the behavioural experiments was done using Microsoft Office Excel™ and the free statistical program R. The statistical tests used to compare different experiments and

solutions were the ANOVA or the non-parametrical Mann-Whitney test, depending on the characteristics of each data set.

Results

CO₂ is a very important and widely spread molecule, not only is it present in important reactions and processes, but it is used as a cue for food tracking and diffusible warning cues.

Many insect species are able to detect CO₂ and are known to use it as a cue. One of the insects that use CO₂ as a cue is the fruit fly *Drosophila melanogaster*. This species is able to detect CO₂ atmospheric levels by specific neuronal cells present in the olfactory system, more precisely in the antennae. They sense CO₂ when its concentration is above normal, when that happens, it elicits aversive behaviours.

In 2007, Fischler *et al.* (2007) found that CO₂ is used by *D. melanogaster* as a cue for food source detection as well, showing appetitive behaviours towards it. The main difference is the population of neurons detecting CO₂ and their location. This neuronal population is in the gustatory system, more precisely in the proboscis, and is able to detect CO₂ released from liquid solutions through carbonation. Not only is the detection different, but the behaviour as well.

How can a single molecule as CO₂ elicit completely different behaviours in the same insect? This may be explained by the projections of both sensory systems, which may project to different brain regions where the different behaviours are initiated.

To track down these regions in adult flies is very difficult, the main reason is the high complexity of the brain and its high number of cells. Therefore, using larvae of the same species will facilitate the definition of brain regions associated with different behaviours. This will be easier with larvae because they have ten times less cells in the brain than the adult. However, the brain structures in larvae are similar to the adult.

Fruit fly larvae have been widely used in behavioural feeding assays and experiments with food preference assays. These assays consisted of a Petri

dishes filled with agar or Agarose, with wells containing solid food patches, where larvae would feed. Each patch had different foods with different dyes, to see differences in preference and to calculate a Preference Index (PI). However, the same kind of experimental setup cannot be used to study preference to carbonation because a liquid solution is needed. Hence, a new setup had to be made in order to allow experiments with liquid solutions and feeding on larvae.

To test preference towards a carbonated solution, two conditions had to be tested. The first condition was the ability of larvae to feed on liquid solutions, therefore a new setup had to be prepared. The second condition was the ability to choose between different liquid solutions. Only then it was possible to test preference towards a carbonated solution.

To test setup conditions and liquid preference, Canton-Special (C-S) wild type flies were used.

Larvae can feed on liquid solutions

To make a new setup, it had to be tested to see if larvae were able to feed on liquid solutions. As in other experimental setups, a Petri dish filled with Agarose 2,5% was used as support for larvae, where they could move freely and the humidity was maintained. Each assay took place in a dark room with constant temperature and humidity, with 30 larvae per assay, for about two hours. These larvae have gone through an overnight starvation period to make sure they are not overfed at the time of the assay. In the middle of those Petri dishes, a cap of 1,5ml eppendorf tubes was used as a container where a solution could be placed without leaking or being absorbed by the Agarose gel (Fig 1A).

The solutions used to test liquid feeding in larvae were Sucrose 0,1M, Yeast extract (0,05g/ml) and distilled water (dH₂O). Sucrose and Yeast Extract were used because they were compounds already known to be appetitive to *D. melanogaster*. We added a red or blue dye to each solution because larvae wander a lot in the experimental setup, and the preference cannot be inferred by position. Therefore, a dyed solution is shown in the digestive tract when larvae feed on them, since larvae are almost transparent the solution is easily seen. The

distilled water solution was used to test feeding on plain water as an indicator of feeding in solutions with no attractive compounds.

In these experiments, the percentage of larvae that could feed on the

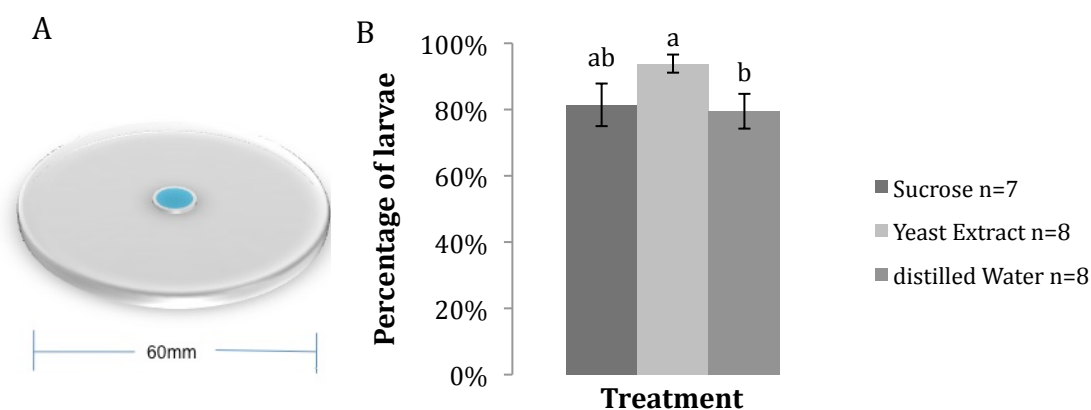


Figure 1 Experimental setup and Percentage of larvae with a dyed solutions per assay.

A. Experimental setup with one container.

B. Graph showing the percentage of larvae that fed on a liquid solution, showing a coloured solution in their digestive tract.

Letter on top of each column represent the statistical level. If they have the same letter, there is no difference between them.

liquid solutions was high (Fig. 1B). The assays with Sucrose showed an average of $81 \pm 15.65\%$ ($n=8$), meaning that 81% of larvae had the dyed solution in their digestive tract, therefore, they could feed on the liquid solution. With Yeast Extract solutions, the number of coloured larvae was the highest registered for this feeding assay, with an average of $94 \pm 7.7\%$ ($n=8$). Since both solutions showed high percentages values, there was no difference between them (Sucrose 0,1M vs Yeast Extract: MWW p-value = 0,09865). A solution of distilled water was used to see if larvae also feed on plain water alone and not only on appetitive solutions after the starvation. These assays showed an average of $79 \pm 14.76\%$ ($n=8$) of coloured larvae per assay, meaning that the majority of larvae feed on water as well. This value for distilled water is high, although it is slightly lower than the other values. There is some difference when compared to Yeas Extract solutions (dH₂O vs Yeast Extract: Mann-Whitney-Wilcoxon [MWW] p-value = 0,03837), but that might due to the low number of assays. dH₂O was compared with Sucrose 0,1M and there was no significant difference seen between solutions (Sucrose 0,1M vs dH₂O: MWW p-value = 0,7711). Both sucrose and Yeast Extract are known to be appetitive for *D. melanogaster* and have some

nutritional level, while dH₂O does not, therefore, the slight difference between solutions is not unexpected nor relevant for the outcome of the experiment.

Larvae can feed on liquid solutions and be scored for the dye present in their digestive tract. It is important to notice that the design of the experimental setup does not hinder movement or normal larval feeding, since their liquid consumption is high. They look healthy and move fast which suggests that the experimental setup has good conditions for behaviour.

Larvae can choose between two liquid solutions

In the previous experiment we saw that larvae can feed on liquid solutions, confirmed by the presence of a dyed solution in the larva digestive tract. Although larvae can feed on liquid solutions, we do not know if they can choose effectively between different solutions, because they are starved overnight and might go into the first container with liquid they get in contact with, changing the outcome of the experiment. Therefore an appetitive solution was tested against distilled water to see if larvae chose effectively between different solutions. Here, dyes are used to see if a choice was done and which of the solutions was the most appealing

To test if larvae can choose between different solutions, a slightly different setup was used (Fig. 2A). Now, the experimental setup is similar to the previously used Petri dish but with two containers instead of one, so that two solutions are used. The preference towards a solution was tested against distilled water. The solutions used were the same as the previous assay, with the addition of a Yeast solution with the same concentration as Yeast Extract, 0,05g/ml. The appetitive solutions used in this liquid preference assay were tested against distilled water, because the objective of this assay was to test its robustness, hence the use of appetitive solutions.

With the data collected from the assays we could calculate a Preference Index (PI), which ranges from -1 to 1, and reflects the positive or negative preference to a liquid solution, respectively.

All 3 conditions showed positive and strong PI values (fig. 2B). This means, larvae have a strong preference toward the appetitive solutions opposed

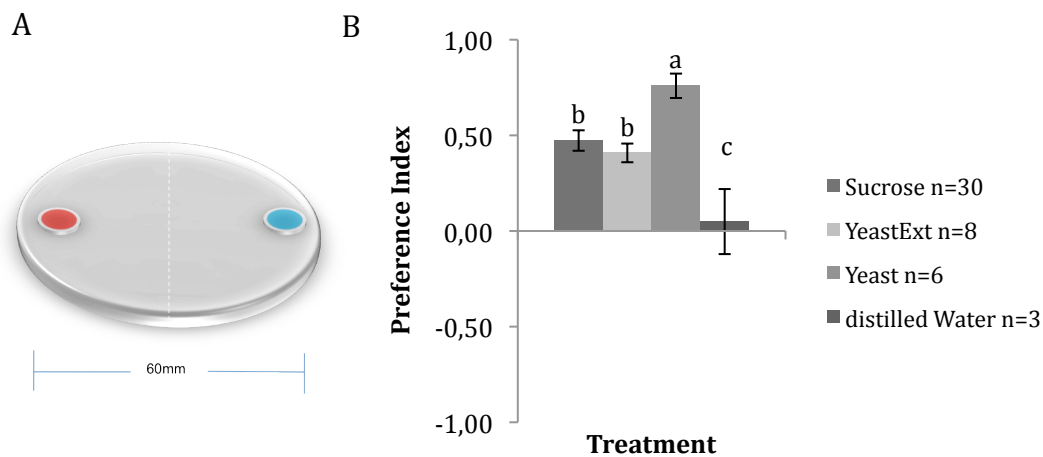


Figure 2. Experimental setup and Preference Assays.

A. Experimental setup used in Feeding Choice assays where two containers are filled with different solutions.

B. In this graph are shown different Preference Indexes for behavioural assays where a choice was made between several solutions vs dH₂O.

Letter on top of each column represent the statistical level. If they have the same letter, there is no difference between them.

to dH₂O. However, larvae did not stay in the first container they found. They are starved but not dehydrated, therefore, they wander around in the experimental setup looking for a food source until they find the best one. Interestingly, due to the fact that larvae are starved, it was expected from them to plunge into the first container with liquid solution, however larvae get in contact with the liquid several times before feeding. This later fact is another reason why dyes are used, because it shows that simple localization of larvae is not enough to assert a preference and a real feeding has to be seen.

PI values in all three solutions reflect a clear preference towards feeding appetitive solutions. Yeast had the highest PI value ($0,76 \pm 0,0637$; Average \pm Standard Error), followed by Sucrose 0,1M ($0,47 \pm 0,0536$) and Yeast Extract ($0,41 \pm 0,0488$; Fig. 2B). Interestingly, in the assays where Yeast solution was used, there is some statistical difference when comparing with the other two treatments (Yeast vs Sucrose 0,1M: ANOVA p-value = 0,0273; Yeast vs Yeast Extract: ANOVA p-value = 0,00079), but this does not go against the experimental plan. When comparing the other two solutions, Sucrose 0,1M vs Yeast Extract, there was no statistical difference (ANOVA p-value = 0,551).

Control assays were made only with dyed distilled water, which indicated that larvae are indifferent distilled water ($0,4924 \pm 0,1752$). All appetitive

solutions showed differences when compared to distilled water, giving emphasis to the indifference to distilled water (dH₂O vs Yeast : ANOVA p-value = 0,0198; dH₂O vs Sucrose 0,1M: ANOVA p-value = 0,0237; dH₂O vs Yeast Extract: ANOVA p-value = 0,0201).

When compared with similar behavioural assays with larvae, these results are reliable and reflect preference towards appetitive behaviours. Although, in other behavioural assays the PI calculation is slightly different and show a smaller scale, therefore they can not be completely compared with these (Ryuda *et al.*, 2008).

Nevertheless, dyes can have an effect on the PI value of each solution, because a difference in preference of dyes might bias the outcome of the experiment (Fig. 3). However, dyes do not have any difference, therefore they do not change the PI value in any of the three solutions used (Sucrose red vs blue: ANOVA p-value = 0,95; Yeast Extract red vs blue: MWW p-value = 0,686; Yeast red vs blue: MWW p-value = 0,7).

With this behavioural assay we can conclude that larvae are able to drink from liquid solutions and can effectively choose between them as well, showing a preference towards one of the appetitive solutions. So we can use this kind of experimental setup to test further preferences for liquid solutions, like a carbonated solution.

This later solution is important because it releases CO₂ through carbonation, that is the conversion of HCO₃⁻ into H₂O and CO₂. CO₂ release has biological relevance because it can be seen as a cue for presence of microorganisms in liquid solutions, such as yeast. And, appetitive behaviours towards solutions with carbon dioxide release were seen in adults of *D. melanogaster*, meaning that this gas has other roles as cue, and is not only associated with aversive behaviours.

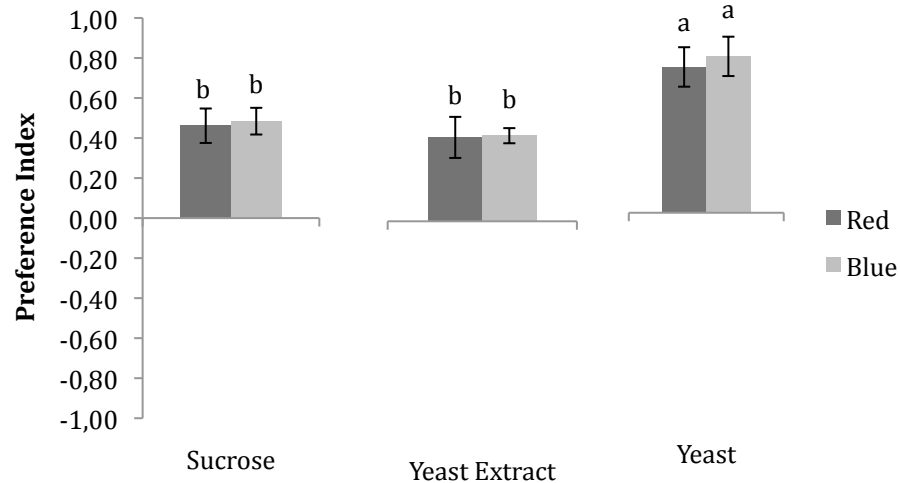


Figure 3. Effect of dyes in the Preference assays.

In this graph are shown the preference index values for each dye in the different treatments.

Letter on top of each column represent the statistical level. If they have the same letter, there is no difference between them.

Larvae show positive preference indexes for carbonated water

In previous experiments we saw that larvae can feed on liquids and effectively choose between them. But we do not know if they show appetitive behaviours towards a carbonated solution as the adult. Therefore, a setup similar to the one used in previous assays was used, like the one shown in Fig 2A.

However, in these behavioural assays the solutions tested were Sodium Bicarbonate solutions that release CO_2 . But they were tested against a buffer solution instead of water to reduce the difference between solutions, because distilled water is very different when compared to a Sodium Bicarbonate solution.

A solution of carbonated water, as mentioned above, releases CO_2 by a process of carbonation, where HCO_3^- is converted into CO_2 . This reaction is present in several reactions and tissues as a pH correction reaction, where HCO_3^- is converted into CO_2 increasing pH levels. When such amount of CO_2 is converted in a solution, it is released to the environment. This reaction mimics CO_2 release by some microorganisms. Therefore, CO_2 can be used by *D. melanogaster* as a possible cue to identify food sources, like yeast or some fruits. Fruits release CO_2 , in decreasing concentration as they are ripening. However, *D.*

melanogaster feed on fruits that are over ripe and fermented, therefore, presence of microorganisms is possible.

Two concentrations of a Sodium Bicarbonate solution were prepared to mimic CO₂ in the behaviour assay, 200mM and 100mM sodium bicarbonate solutions. To each one of the 200mM and 100mM bicarbonate solutions, a 0,5M Phosphate buffer was added, 6,5 and 6,0 pH, respectively, in order to maintain a low pH, so that a constant amount of CO₂ is released (Fischler *et al.*, 2007).

During initial assays, a possible accumulation of CO₂ in the experimental setup was brought up. Therefore, to exclude this possibility, behavioural assays were done with or without lids in the Petri dishes, and in the later, the plastic lid was replaced by a mesh that did not allow larva to leave the experimental Petri dishes, but allowed diffusion of any gas being released from either solution. Preventing CO₂ accumulation in the experimental setup. The alteration to the experimental setup had to be tested for possible differences. From now on assays with or without lid will be referred to as Closed and Open experimental assays. This comparison between setups excludes a potential olfactory contribution, by accumulation of CO₂ in the experimental setup, which could change the outcome of the experiment, altering the behaviour shown by larvae.

Both setups were tested for the highest solution concentration. The PI for C-S flies in Closed assays ($0,571 \pm 0,06952$; Average \pm Standard Error) was a little bit lower than the PI from Open setup assays ($0,608 \pm 0,0583$; Fig 4A). Although the PIs are different, there was no statistical difference between them (ANOVA p-value = 0,937). Hence, using slightly different setups does not influence the outcome of the assay, meaning that there is no olfactory contribution by accumulation of CO₂ in the setup.

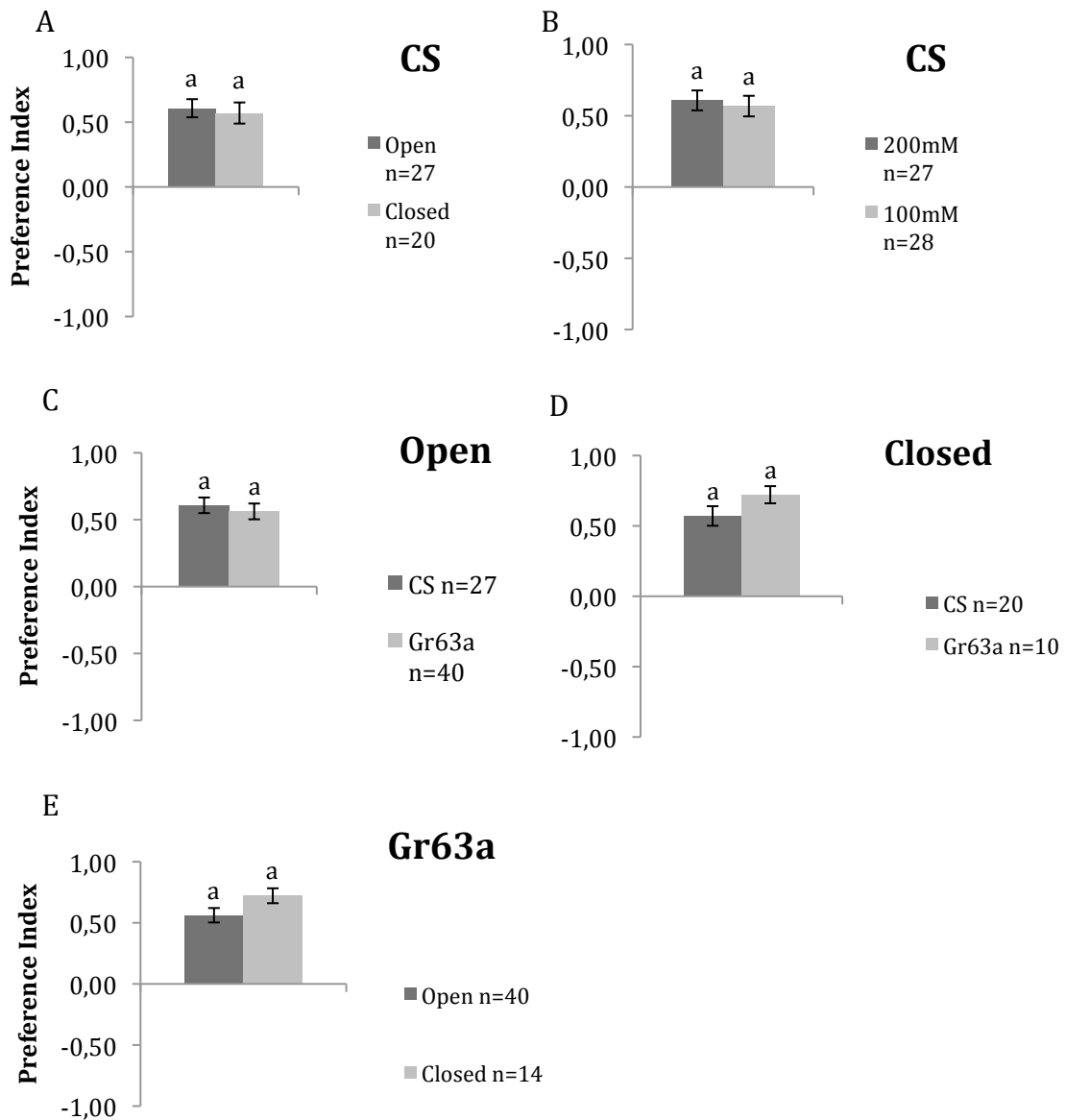


Figure 4. Bicarbonate solution preference assays.

(A) PI values for the behaviour assays with C-S flies to test a slight difference in experimental setup using a lid or a lidless petri dish, to see if the design of the experiment creates any effect possible of biasing the results.

(B) Preference Index values for two concentration solutions of carbonated water in behavioural assays with wild-type flies.

(C) Comparison between fly strains using an Open dish behaviour assay with a 200mM carbonated solution.

(D) Comparison between fly strains using an Closed dish behaviour assay with a 200mM carbonated solution.

(E) Preference Index values for Gr63a¹ with Open and Closed Petri dishes.

Letter on top of each column represent the statistical level. If they have the same letter, there is no difference.

After testing if there was any difference using slightly different setups, different concentrations of a carbonated solution were tested. Concentrations of 200mM and 100mM were tested for larval preference to see if larva respond differently towards different concentrations of carbonated solutions. PI values obtained from were strong and fairly high, both when using 200mM ($0,608 \pm$

0,0583) and 100mM ($0,568 \pm 0,0722$) (Fig. 4B). Interestingly, 100mM PI values were as high as 200mM values, when a discrepancy was expected due to a two-fold difference in concentration. However, since values are so similar, there is no statistical difference was seen between them (MWW p-value = 0,9839).

PI values for the carbonated solution assays were high, showing that larvae have a positive preference towards a carbonated solution as opposed to a plain solution. Therefore, larvae show appetitive behaviours towards carbonated solution, like the adults. However, unlike the adults, larvae cannot detect a difference between the tested concentrations.

There is no olfactory contribution of CO₂ to the preference index

It was seen in the previous assay that larvae have appetitive behaviours to a carbonated solution that releases CO₂. This gas is a possible cue for the detection of food sources because microorganisms they feed on release it, therefore, these solutions elicit appetitive behaviours in flies because they are associated with solution where microorganisms are present. However, since CO₂ elicits aversive behaviours in flies when detected by the olfactory system, there might be an olfactory contribution when using carbonated solutions.

Therefore, another fly line was used to check for possible olfactory contributions in the experimental setup. The Gr63a¹ fly line was used with that purpose because it is a fly line characterized by a mutation in Gr63a¹, one of the two known receptors for CO₂ in the olfactory sensory neurons (Suh *et al.*, 2004). If one these receptor is mutated, flies are unable to sense CO₂. These CO₂ receptors neurons must have both receptors present and working to sense CO₂. Hence, a mutation in the Gr63a CO₂ receptor makes flies unable to sense CO₂, even if this gas is present at high concentrations.

Therefore, this fly line was used to check for possible olfactory contributions that can change the preference for carbonated solutions. However, it is unknown if the olfactory system inhibits the gustatory system or vice-versa.

The preference assays with this fly line were done in the same conditions as C-S flies, although, only the highest concentration of 200mM was tested. Using the experimental setup shown in Fig. 2A. The presence of lid was also tested,

because in this case the olfactory contribution of CO₂ to the preference assay is absent. Therefore, both Open and Closed assays were done. Closed assays with Gr63a¹ showed a surprisingly high PI value ($0,722 \pm 0,06099$), however, this PI value is not different from Gr63a¹ Open assays ($0,562 \pm 0,05907$; Fig. 4E). Like C-S assays, there is no statistical difference between setups (MWW p-value = 0,1439). Therefore, there is no difference between experimental setups.

PI values from Open assay revealed a strong preference to carbonated water ($0,562 \pm 0,05907$), like the values seen for C-S assays ($0,608 \pm 0,0583$). Closed Gr63a¹ behaviour assays were high ($0,722 \pm 0,06099$), however, there was no difference from the Closed assay with C-S flies ($0,571 \pm 0,06952$). Therefore, these results suggest that there is no difference seen between fly strains, both in Open assays (MWW p-value = 0,8106; Fig. 4C) and in Closed assays (ANOVA p-value = 0,1324; Fig. 4D).

These results suggest that there is no olfactory contribution of CO₂ to the outcome of the experiment. Therefore, the levels of CO₂ released by the carbonated are so low that larvae cannot detect them. Resulting in the absence of aversive behaviours towards CO₂.

Dye contribution for the preference index was once again checked for a possible bias because the solutions are different and have different reagents. In previous assays dyes do not show any difference, however, since we are using new different reagents, there might be some reactions that were not expected. Therefore, difference between dyes was checked because these could change the result of the experiment. A difference was seen in C-S Open Petri dish assays, with a slight increase to the blue bicarbonate solution PI value. In addition, another slight increase was seen to the red dye in Closed Gr63a¹. However, this does not influence the outcome of the experiment, because PI values continue to show appetitive preference towards carbonated water, therefore, it does not against the behavioural hypothesis.

Discussion

CO₂ molecule has a high biological importance because it is present in many processes and reactions. Some of them are even crucial for the animal to survive. Since CO₂ is a molecule present everywhere, it can have many roles, in some environments it serves as a cue, in others it can even be part of important reactions and processes. Although it has no specific role in metabolism, this gas is a product of this process, therefore, it is released by many organisms.

Insects for instance, use CO₂ in various ways. They can use it as a cue to identify and search for food sources, like moths or mosquitoes. Moths use CO₂ as a cue to detect plants or other food sources they feed on. In the other hand, mosquitoes can detect CO₂ plumes release by other animals as product of their metabolism. Since they feed on blood, they use CO₂ as an olfactory cue for food detection. In moths and mosquitoes the CO₂ receptors are in the maxillary palps, which are structures usually associated with feeding.

In *Drosophila melanogaster*, or the commonly called fruit fly, the scenario is different. CO₂ is detected by receptors in the antennae instead of the maxillary palps. When CO₂ is detected in concentrations higher than normal, flies show aversive behaviours. Therefore, CO₂ is a possible environmental cue. It is important to know that stressed flies release CO₂ as a component of their stress odorant, giving emphasis to the aversive behaviours towards this gas. Until 2007, only the negative effects of CO₂ were known in fruit fly behaviour, but this changed when Fischler *et al.*, (2007) found that flies can show attractive behaviours to a solution that release CO₂. They found that there are specific cells responsible for the detection of CO₂ released by carbonation in a liquid solution. Interestingly, these cells are in the proboscis and the receptors for CO₂ are different from the olfactory receptors for the same gas.

In the olfactory system it is known that the ORN project to the AL, the first organ of information processing, and then after to the MB and the LH. Further projections of the neurons are unknown due to the complexity of the brain and the high number of neuronal cells.

In the gustatory system, the scenario is similar. The only known projections go from the GRN into the SOG, the first order organ of information processing. From here onward, the situation is the same as in the olfactory system. Higher projections are unknown and too complex to define.

That's why larvae were used in this work. They have a lower number of neurons and cells but the structure of the brain is similar to the adult brain. Using a simpler model, considered by some as an elementary model to study the olfactory system (Ramaekers *et al.*, 2005), tracing projections might be easier. Therefore, it might be possible to trace regions in the brain responsible for basic behaviours like appetitive or aversive behaviours.

However, *D. melanogaster* adult flies are known to show aversive behaviour to CO₂ in its gas phase, while showing appetitive behaviours to a solution that releases CO₂. Larvae are known to show aversive behaviours to CO₂ in its gas phase as well, but no behaviours are known as response to a carbonated solution.

Therefore, larval behaviour to carbonated solutions had to be tested. However, feeding assays in larvae described until now, always use solid food sources to test preferences. But to test preference to a carbonated solution, these assays with solid food do not work. Therefore, to test preference to carbonated solutions, a feeding assay with liquids has to be tested.

First we had to see if larvae, from wild-type populations (C-S), could feed on liquids. To test this possibility appetitive solutions for *D. melanogaster* were used. Although, some changes had to be done in the experimental setup, to be able to support liquid solutions. Thus, the new experimental setup had one container for the solution instead of patches with solid food. For every solution a dye was added to see if larvae fed on those solutions, instead of relying on the position on the Petri dish to infer preference.

These assays had high numbers of larvae coloured with the appetitive solutions. Therefore we can say that larvae can feed on liquid solution with no difficulty, since a high number of larvae had the appetitive solution in their digestive tract.

From this experiments we know that larvae can feed on liquids without being hindered by the design of the experimental assay. Therefore, the next step

is to see if larvae can effectively choose between two different solutions. This new experimental setup had two containers instead of one, so that two solutions could be tested instead of one. Because larvae were starved overnight, they could go into the first solution they saw and be stuck in that container. However, this does not happen, showing the experimental setup does not hinder their movement. In addition, containers do not hinder larvae, because they can come out of the container with no difficulty. Due to wandering, the preference in the experimental assay cannot be calculated using positioning of larvae, which would lead to an incorrect interpretation of the preference towards a liquid. Therefore, dyes were added to the solution to see where larvae fed. Results of these experimental assays showed that larvae could feed on liquids and effectively choose between different solutions. In these assays, appetitive solutions were used because the objective was to see if larvae could feed on liquids and choose between different liquids. Data was collected as Preference Indexes, an index calculated based on the amount of larvae with coloured solutions in their bellies (for more detailed information see Methods). If two appetitive solutions were used in the same experimental setup, there might be an outcome hard to interpret and since the objective of this experimental behaviour is to test a choice between solutions, there is no need to complicate the experimental assay. All solutions in this assay had positive PIs with relatively high levels. When control experiments were made with distilled water in both wells, the PI value was close to zero, indicating that there is no preference. All taken together indicate that larvae can feed on liquids and even effectively choose between two liquid solutions. Therefore, further experimental assays can be done with liquid solutions. Another fact to take into consideration is a possible effect of dyes, because we are adding dyes to a solution, this could alter the outcome of the experiment. However, there was no difference seen between dyes, and the preference indexes did not change.

Since larvae can feed on liquids and even effectively choose between solutions, we can do behaviour assays using carbonated water, to see which kind of behaviour larvae show when in the presence of these solutions. In these assays with carbonated solutions, a solution of Sodium Bicarbonate was used to mimic the CO₂ release seen in solutions with microorganisms. We didn't use a

solution with live yeast because it would bias the outcome of the assay by adding other olfactory and gustatory contributions to the assay. Another reason why a solution of live Yeast was not used, regarded the amount of CO₂ released, which can be controlled with a solution of sodium bicarbonate and a gas meter. But cannot be controlled in a solution with live yeast. In these assays with carbonates solutions, the control solution was a buffer instead of water, because water was too different from the carbonated solution, what would not help the experimental plan. Since the carbonated solution released CO₂, there might be an olfactory contribution from this gas, what would change the preference of the experimental assay. Therefore, two slightly different experimental setups were used, one with the lid and the other without the lid. Petri with a mesh instead of the lid, allowed the CO₂ released to diffuse to the environment. Not allowing accumulation in the experimental setup, that could change the result of the assay. These setups were called Open, for the assay with no lid, and Closed, for the assay with the lid on top. However, after doing behavioural assays in both Open and Closed setups, we saw appetitive behaviours towards carbonated water in both, with no difference between them. What suggested that there was no olfactory contribution to the outcome. Since there was no contribution of the olfactory system, larvae were not sensing CO₂. This could be due to low concentrations of CO₂ barely detectable by larvae.

Different concentrations were tested for behaviour as well. These concentrations were 100mM and 200mM of a Sodium Bicarbonate solution. In these assays, the preference was very similar, almost identical what suggests that larvae cannot detect a difference between them. Therefore, the CO₂ concentration is very low in both solutions, what makes their discrimination very difficult. From the results we can assume that larvae cannot see any difference between these concentrations.

To exclude the olfactory contribution to the preference index, another fly line was used, The Gr63a¹ fly line. This fly line is unable to detect CO₂ through olfactory pathways because it lacks one of the CO₂ receptor in the olfactory sensory neurons. Therefore, using this fly line we can completely exclude the olfactory contribution to the behavioural assay. However, in these experimental assays with Gr63a¹ we saw high preference levels for the carbonated solutions,

what was expected, but no difference was seen between strain. Both in Open and Closed setups, meaning that there is no CO₂ olfactory contribution to the behaviour shown by larvae. And that CO₂ had no olfactory contribution to these experimental behaviours. Therefore we can conclude that larvae are attracted to solutions of carbonated water as the adults. However, the effect of dyes was checked for a possible contribution to the experimental assay. A difference between dyes was seen in Open C-S assays, with blue having a slightly higher preference index, however, since both assays show a positive preference, this does not go against our finding. But, in Gr63a¹ assays, the dyes showed some difference as well. Although, this time it was on the Closed assays and as an increase in preference for the Red dye. However, the values are both positive and the behaviour is the same, larvae show appetitive behaviours to the carbonated solutions, therefore, these results do not go against our findings.

As a conclusion we can say that larvae from *D. melanogaster* can be used to test feeding assays with liquid solution, because they can feed on liquids and effectively choose between solution. Therefore, we could do behavioural assays with carbonated solutions where they showed appetitive behaviours, like the adults. Although, to confirm this possibility another set of experiments can be done, inactivating or killing the cell line responsible for CO₂ detection in the gustatory system. To confirm that those cells are responsible for CO₂ detection. But, this might be a project for future work to further study the detection of carbonation by larvae. However, with these results it is possible to start thinking of ways to trace CO₂ projections into higher order organs in the olfactory and gustatory system using larvae, because they have the same structural organization with the reduced cell numbers. While showing the different behaviours elicited by CO₂. That suggests that there are different brain regions responsible where different behaviours are elicited, even by the same molecule.

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